UNIVERSITY OF PENNSYLVANIA

A STUDY OF HEMOPHILUS INFLUENZÆ

BY

MARY JARDINE EVANS

A THESIS

IN BACTERIOLOGY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PHILADELPHIA

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A STUDY OF HEMOPHILUS INFLUENZÆ.*

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In September, 1926, the problem was started of grouping serologically Hemophilus influenzæ by means of agglutination and precipitin reactions, and of testing the virulence of these strains, thereby to determine if there was any correlation between serologic grouping and strain-virulence.

Preliminary Experiments. Twenty-eight strains of Hemophilus influenzæ were isolated from cultures of the pharynx, sputum, pleural fluids and one spinal fluid. The virulence of these microörganisms varied when injected into the peritoneal cavity of mice. In eight strains, 1 billion microörganisms killed the animals within eighteen hours, and in two strains the fatal dose in this time was $\frac{1}{2}$ billion microörganisms. All these strains were considered virulent. The eighteen other strains, in which 2 billion of the microörganisms did not kill mice after seventy-two hours were considered avirulent. Immune monovalent sera were prepared in rabbits for eight of these virulent strains.

In January, 1927, a strain of Hemophilus influenzæ was isolated from the sputum of a patient suffering with acute influenza, on the third day of the disease. One-eighth of a billion microörganisms of this strain, designated as the Hammett strain, killed mice within eight hours. Because this strain appeared unusually virulent for mice, it was tested in rabbits. One-quarter of a billion living microörganisms of this strain injected intravenously, killed the rabbit over night, and a pure culture of it was recovered from the lungs, peritoneal exudate, and the heart blood.

Experimental Work. A series of experiments was then started to test whether the pathogenic factor of this strain was a toxic agent, which might be demonstrated in the filtrates of cultures. Parallel experiments were conducted with other strains of Hemophilus influenzæ, isolated from patients in the Philadelphia General Hospital. This work was divided in the following manner:

* A Thesis in Bacteriology. Presented to the Faculty of the Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(*a*) Cultural experiments and virulence tests with twenty-three strains of Hemophilus influenzæ isolated from human sources.

(*b*) Experiments with culture filtrates of Hemophilus influenzæ.

(*c*) Rabbit experiments.

(*d*) Biologic studies on Hemophilus influenzæ.

(*e*) Serologic reactions with immune serum.

(*f*) Therapeutic tests of Hemophilus influenzæ (Hammett strain) immune serum.

(*a*) **Cultural Experiments and Virulence Tests with Twenty-three Strains of Hemophilus Influenzæ.** 1. *Preservation of Stock Cultures of Hemophilus influenzæ.* Because of the observation of workers[1] that Hemophilus influenzæ quickly loses its virulence when kept on artificial media, a method of preserving the virulence of stock cultures was sought. The culture medium employed in this study was brown blood broth, that is, infusion broth of pH 7.6 to 7.8 to which approximately 7 per cent by volume of sterile defibrinated blood was added. This was then heated until the blood became a rich brown color. The medium was tubed and incubated at 37.5° C. to test its sterility before use.

When cultures were first isolated from patients or from animals, transplants were made every three or four days, but as the cultures became adapted to the medium, transfers were necessary only every three weeks. At the time of transplanting, the cultures were also tested for purity by plating on brown blood agar. Those used for experimental work were always kept in the brown blood-broth medium, at room temperature (18° to 20° C.). It was found, in the course of this study, that cultures of Hemophilus influenzæ though virulent as long as grown in brown blood broth, when transplanted to a solid medium for four or five generations, became avirulent. When stock cultures were kept at ice-box temperature, loss of virulence was more rapid than at room temperature. In the two years that the Hammett strain has been under observation, it has lost little of its original virulence. The cultures of the Hammett strain reclaimed after animal passage, and also strains from other sources, have all been maintained in the same manner and have remained virulent.

Thus, the virulence of a culture of Hemophilus influenzæ has been maintained over a period of two years, all strains studied being cultivated and stored in a fluid medium, that is, brown blood broth, at room temperature (18° to 20° C.). It appears that the important points in this method of cultivation are the composition of the medium and the temperature at which the cultures are kept.

2. *Virulence Tests.* In testing virulence, 0.2 cc. of a brown blood broth culture of Hemophilus influenzæ was streaked on a brown blood-agar plate and incubated at 37.5° C. After eighteen hours the growth was washed from the plate with infusion broth, since it was found that an exposure of ten minutes to physiologic saline solution destroyed the virulence. The suspension was centrifuged

at low speed for a half minute to remove agar particles, and diluted to contain 1 billion microörganisms per cubic centimeter using infusion broth as the diluent. It was then injected intraperitoneally into the mouse. Estimation of the virulence of a strain was based upon the numbers of microörganisms and the time required to cause the death of the mouse. Of the twenty-three strains studied, thirteen were virulent and ten were avirulent.

(*b*) **Experiments with Culture Filtrates of Hemophilus Influenzæ.** 1. *Preparation of Filtrates.* Filtrates were obtained of the brown blood-broth cultures after growing the bacteria for eighteen hours at 37.5° C. After centrifuging the culture to throw down the heavy particles of the medium, the supernatant fluid was filtered through a Grade N. Berkefeld filter. For the Hammett strain, it was found that this filtrate would kill mice within eighteen hours when 2 cc. was injected intraperitoneally, and rabbits when 3 cc. was injected intravenously. Filtrates from the other strains had to be used in larger volumes to produce corresponding effects.

2. *Skin Reactions with the Culture Filtrate of the Hammett Strain.* The culture filtrate of the Hammett strain prepared as described above in dilutions of from 1 to 100 to 1 to 7000 for skin tests upon individuals volunteering for the study. Control tests were made with filtered brown blood broth and normal horse serum similarly diluted. Sixty individuals were tested using 0.1 cc. of the dilutions of filtrate injected intradermally. The results were read after six and eighteen hours. After forty-eight hours the local reactions had faded materially. The typical reactions consisted of an erythema or a pink papule around the point of injection, and at times the erythema was surrounded with a blanched area. An erythematous reaction of less than 5 mm. in diameter was regarded as negative. Table I shows the results of these tests, the measurements of the local erythema being recorded in millimeters.

In the first group of 60 nonfebrile patients in the hospital wards, 42 gave positive skin reactions, the remaining were negative, or gave positive reactions in the control. Several individuals who gave negative results gave histories of exposure to influenza patients in the 1918 epidemic without contracting the disease at that time. Others had had very severe attacks of influenza in 1918 and recovered. One of these individuals had had a typical attack of influenza in the epidemic of 1889–1890 and though thoroughly exposed in the epidemic of 1918 did not contract the disease. In the second group of 75 normal volunteers, representing students, interns, nurses and laboratory workers, 51 gave positive and 24 negative skin reactions, or approximately 68 per cent reacted positively.

The results of the foregoing experiments show: (1) In virulent cultures of Hemophilus influenzæ there appears to be a toxic agent capable of causing death of white mice when injected intraperitoneally. (2) This toxic agent is not found in filtrates of avirulent

strains. (3) Culture filtrates of virulent strains of Hemophilus influenzæ produced skin reactions in 68 per cent of humans when employed in dilutions up to 1 to 7000. This test seems to be analogous to the Schick or Dick test and appears to be of such a nature that it may be of value in determining susceptibility to infection with Hemophilus influenzæ, and should be further investigated.

TABLE I.—RESULTS OF SKIN TESTS ON HUMANS, PERFORMED WITH FILTRATE OF CULTURES OF HEMOPHILUS INFLUENZÆ (HAMMETT STRAIN), THE READINGS BEING RECORDED IN MILLIMETERS.

Patient.	1 to 100.	1 to 1000.	1 to 2000.	1 to 3000.	1 to 4000.	1 to 5000.	1 to 6000.	1 to 7000.	Control.*
1	20 x 20	20 x 18	8 x 10	7 x 7	—	—	—	—	—
2	18 x 14	15 x 15	15 x 15	12 x 10	10 x 10	7 x 5	—	—	—
3	22 x 15	18 x 20	11 x 12	10 x 10	5 x 7	—	—	—	—
4	18 x 25	18 x 20	20 x 20	20 x 15	10 x 10	—	—	—	—
5	23 x 22	20 x 18	8 x 7	9 x 10	10 x 10	5 x 5	—	—	—
6	19 x 22	16 x 26	14 x 22	14 x 20	10 x 10	10 x 10	—	—	—
7	30 x 25	26 x 22	14 x 26	11 x 5	9 x 6	5 x 6	5 x 5	—	—
8	17 x 20	14 x 15	11 x 22	10 x 15	9 x 5	—	—	—	—
9	14 x 11	13 x 10	10 x 11	5 x 5	—	—	—	—	—
10	...	19 x 24	17 x 18	16 x 16	16 x 15	6 x 6	—	—	—
11	...	18 x 20	11 x 11	9 x 10	9 x 10	7 x 6	—	—	—
12	...	22 x 22	16 x 14	16 x 15	12 x 11	5 x 5	—	—	—
13	...	26 x 22	20 x 18	15 x 16	12 x 12	10 x 10	—	—	—
14	...	18 x 20	15 x 19	15 x 19	15 x 17	10 x 10	—	—	—
15	...	10 x 15	8 x 8	8 x 7	7 x 6	8 x 6	5 x 6	—	—
16	...	20 x 22	19 x 19	16 x 17	11 x 10	11 x 5	5 x 5	—	—
17	...	17 x 22	17 x 15	16 x 10	14 x 14	10 x 10	—	—	—
18	...	17 x 17	15 x 12	15 x 12	15 x 10	5 x 5	—	—	—
19	...	16 x 18	11 x 14	10 x 13	8 x 6	3 x 3	—	—	—
20	...	14 x 15	...	...	...	14 x 13	12 x 14	9 x 7	—
21	...	10 x 14	...	...	...	11 x 9	7 x 7	—	—
22	...	12 x 10	...	...	...	10 x 10	9 x 10	7 x 6	—
23	...	12 x 16	...	...	...	14 x 14	14 x 16	9 x 7	—
24	...	10 x 10	...	...	...	9 x 9	10 x 7	6 x 6	—
25	...	...	...	...	...	15 x 16	10 x 11	6 x 6	—
26	...	...	...	...	...	10 x 8	8 x 6	4 x 5	—
27	...	...	...	...	...	11 x 12	10 x 7	4 x 9	—
28	...	...	...	...	...	14 x 12	11 x 9	7 x 4	—
29	...	...	...	...	...	9 x 10	6 x 7	5 x 8	—
30	...	...	...	...	...	25 x 27	14 x 11	9 x 4	—
31	...	...	...	...	...	11 x 14	9 x 6	4 x 4	—
32	...	...	...	...	...	10 x 8	11 x 8	8 x 5	—
33	...	...	...	...	...	13 x 12	10 x 9	8 x 6	—
34	...	...	...	...	...	16 x 18	12 x 12	9 x 9	—
35	...	...	...	...	...	8 x 8	6 x 8	4 x 4	—
36	...	...	...	...	...	18 x 20	16 x 14	12 x 12	—
37	...	...	...	...	...	14 x 16	8 x 8	4 x 6	—
38	...	...	...	...	...	20 x 20	20 x 16	12 x 16	—
39	...	...	...	...	...	10 x 12	10 x 10	6 x 4	—
40	...	...	...	...	...	8 x 10	8 x 6	4 x 4	—
41	...	...	...	...	...	22 x 18	12 x 12	6 x 6	—
42	...	...	...	...	...	24 x 20	20 x 16	12 x 14	—

The figures at the top of the chart, that is, 1 to 100, 1 to 1000, 1 to 2000 and so forth, represent the dilution of the filtrate used in performing the tests.

* Control, uninoculated filtered brown blood broth and normal horse serum.

(*c*) **Experiments on Rabbits.** As mentioned previously, experiments were carried out on rabbits with Hemophilus influenzæ (Hammett strain). Twenty-three animals were used, 8 for animal passages of the Hammett strain, 7 for culture filtrate injections, 6 for exposure in testing contact infection of animals. Two were used for the implantation in the abdominal cavity of sealed collodion sacs containing culture or filtrate of Hemophilus influenzæ.

1. *Effects of Intravenous Injection.*

Protocols. Rabbit 1. Weight, 2100 gm. Injected with 5 cc. of the supernatant fluid from a centrifugated eighteen-hour culture, of the Hammett strain. The next day the animal was ill; on the fourth day dull and listless; on the fifth day it developed paralysis of the hind legs, diarrhea, and purulent conjunctivitis; on the eighth day the paralysis was very marked, accompanied by spasmodic movements, and the animal died several hours later.

Necropsy. The pleural cavity contained pus. There was an acute purulent pericarditis. The smaller bronchial tubes were filled with pus. The lungs had many small pin-point hemorrhages. There was an acute purulent conjunctivitis. The intestines were distended, the liver was dark and friable, and the kidneys were very pale.

Pure cultures of the microörganisms were recovered from the conjunctiva, from the bronchii of both lungs, from the pleural cavity and from the heart blood.

Rabbits 2 and 3. Weight, 2520 and 2780 gm. Injected with 1 and 2 billion respectively of living microörganisms (Hammett strain). The next morning, both rabbits were dead.

Necropsy. The lungs were hemorrhagic with serous exudate into the plural sac, the heart was pale and there were subcutaneous hemorrhages. The lower lobes of the lungs of both animals were more inflamed than the upper lobes. Hemophilus influenzæ was recovered from the lower lobes of the lungs of both animals.

Rabbit 4. Weight, 3040 gm. Injected intravenously with $\frac{1}{8}$ billion living microörganisms. The next day the animal was quiet and listless. On the second day the animal developed bloody diarrhea and died. Pure cultures of Hemophilus influenzæ were recovered from both lungs and the heart blood.

The foregoing experiments with Rabbits 1 to 4 inclusive show the pathogenicity of the Hammett strain for rabbits. One billion living organisms injected intravenously, killed in eighteen hours. Amounts as little as $\frac{1}{8}$ billion proved fatal to rabbits within forty-eight hours. Acute inflammation of the lungs was produced with pin-point hemorrhages, such as is frequently seen in humans dying of influenza. Acute dilatation of the heart at times accompanied by a purulent pericarditis was noted commonly. A serous exudate was constant in the pleural sacs, and Hemophilus influenzæ was readily recovered in cultures from the heart blood and lungs.

2. *Effects of Subcutaneous Doses.*

Protocols. Rabbit 5. Weight, 2375 gm. Injected subcutaneously with 1 billion living microörganisms and intravenously with 1 cc. of culture filtrate (Hammett strain). The animal died on the third day.

Necropsy. The lungs and peritoneal cavity contained a serous exudate. The lungs showed pin-point hemorrhages. One side of the left lung was completely hemorrhagic. The heart was enlarged and there was pus in the pericardial sac. Pure cultures of Hemophilus influenzæ were obtained from the peritoneal cavity, from the lungs, heart blood and liver.

Rabbit 6. Weight, 2640 gm. Injected subcutaneously with $\frac{1}{4}$ billion living microörganisms of the Hammett strain. Two days later a swelling developed at the point of inoculation. The next day the swelling extended over approximately four square inches of the abdomen. On the fourth day the swollen area opened at the point of inoculation. A small incision was made and cultures taken. These showed pure cultures of Hemophilus influenzæ. By the eighth day the area of infection was extending and by the tenth day the abscess had broken down and a thick, yellow ropy pus exuded. On the fourteenth day the opposite side of the abscess was opened and allowed to drain. On the twenty-fourth day a pure culture of Hemophilus influenzæ was obtained from the wound. In this animal a purulent ulcerating lesion from which Hemophilus influenzæ could be cultured at will persisted until the death of the animal, eight months later. This experiment indicates that the Hemophilus influenzæ organism when injected subcutaneously is not as rapidly fatal as when injected intravenously. Rabbit 6 was used for subsequent experiments.

3. *Results of Experiments for Immunization.*

Protocols. Rabbit 7. Weight, 2200 gm. Animal received subcutaneously $\frac{1}{4}$ billion killed organisms of the Hammett strain on the first, third, fifth and twenty-third days. Injections were not made between the fifth and twenty-third days because of loss of weight of the animal. On the twenty-fifth day $\frac{1}{2}$ billion killed organisms were injected subcutaneously. On the thirty-seventh day $\frac{1}{4}$ billion living organisms were injected subcutaneously. The agglutinin titer of the serum taken just prior to the injection of the living organisms on the thirty-seventh day was 1 to 160. The animal died eighteen days after receiving living organisms. It was ailing for several days with a small lump at the point of inoculation.

Necropsy. The lungs were hemorrhagic with dark pin-point hemorrhages throughout. A serous exudate was present in the pleural cavity, and the heart was much enlarged. Subcutaneous capillary hemorrhages were also present. Pure cultures of Hemophilus influenzæ were recovered from the pus of the point of inoculation, from the lungs, the heart blood and the peritoneal exudate.

Rabbit 8. Weight, 3140 gm. Animal received subcutaneously $\frac{1}{4}$ billion killed organisms of the Hammett strain on the first and third days, and $\frac{1}{2}$ billion killed organisms on the sixth, eighth, tenth and twelfth days. On the fourteenth, eighteenth and twenty-second days, $\frac{1}{16}$ billion living organisms were injected subcutaneously, and $\frac{1}{4}$ billion on the twenty-fourth and thirty-first days. The next day the agglutinin titer of the serum was 1 to 320. The animal developed an abscess at the point of inoculation of the living cultures and was used for subsequent contact experiments. (See Section Four.) The rabbit died after sixty-eight days, presenting throughout this period an abscess at the point of inoculation. No autopsy was performed.

Attempts at immunization with the Hammett strain failed, apparently because of the extreme virulence of this strain. The animals lost weight and when inoculated with living organisms, even following previous inoculations with killed organisms, became infected, and finally death ensued.

4. *Infection of Normal Rabbits Through Exposure to Infected Animals.* Normal rabbits were placed in cages with rabbits previously infected with Hemophilus influenzæ, to determine if spontaneous infection would occur in normal rabbits as a result of contact. By referring to Section three it will be noted that Rabbits 6 and 8 had as a result of experimentation developed chronic abscesses and hence were utilized in this phase of the work to determine if through contact with these rabbits normal rabbits might become infected. The procedure and the results of experiments were as follows:

Rabbit 9. A normal rabbit was placed in a cage with Rabbit 6. Two days latter Rabbit 9 was listless and died nine days later.

Necropsy. The lungs were hemorrhagic with emphysemic patches at the edges. The small bronchi were filled with pus. The heart was enlarged and there was abundant exudate in the peritoneal cavity. Hemophilus influenzæ was recovered from the peritoneal fluid, heart blood and lungs.

As a control, Rabbit 10, a normal rabbit was exposed to Rabbit 13 which had previously received 2 cc. of culture filtrate intravenously to see whether a normal rabbit would become infected from a rabbit receiving filtrate. After five days, no infection developed, in either rabbit. Rabbit 10 was then placed in a cage with infected Rabbit 6 and the result of this experiment was as follows.

Rabbit 10. A normal rabbit was placed in a cage with Rabbit 6. The animal appeared to be ill after two days and died after eleven days.

Necropsy. The lower lobes of the lungs were hemorrhagic and the upper lobes contained pin-point hemorrhages throughout. Hemophilus influenzæ was recovered from the heart blood, and the lungs.

Rabbit 11. A normal rabbit was placed in a cage with Rabbit 6. Rabbit 11 died in ten days. At autopsy, it showed a serous pleural exudate with inflamed and hemorrhagic lungs. Hemophilus influenzæ was recovered from the heart blood, lungs and pleural exudate.

The above experiments show that infection may be transmitted by contact when normal rabbits are exposed in the same cage with infected rabbits, the infection appearing in two days and death occurring within about ten days.

5. *Infection Occurring in Rabbits First Inoculated with a Sublethal Injection of Filtrate and then Exposed to Infected Rabbits with Open Lesions.* Since the experiments in Section Four shows that normal rabbits become infected when brought in contact with rabbits previously infected with Hemophilus influenzæ and having open lesions, experiments were performed to determine if rabbits previously injected with culture filtrate would when brought in contact with infected rabbits, become infected. The procedure and results were as follows:

Rabbit 12. A normal rabbit after receiving 5 cc. of culture filtrate of the Hammett strain subcutaneously was placed immediately in a cage with infected Rabbit 8.*

* As will be shown later, 3 cc. of culture filtrate of the Hammett strain injected intravenously killed in eighteen hours. However, it was found that rabbits withstood larger injections of culture filtrate subcutaneously without succumbing.

After four days of this contact, the animal developed an abscess behind the ear, from which Hemophilus influenzæ was recovered in pure culture. The animal died on the eighth day after exposure. At autopsy, the lungs were hemorrhagic with small patches of emphysema and the heart was enlarged. Hemophilus influenzæ was recovered from the abscess behind the ear, the lungs and heart blood.

Rabbit 13. Received 2 cc. of culture filtrate intravenously and was placed with Rabbit 8. He developed a purulent conjunctivitis and died on the fifth day. An autopsy was performed and Hemophilus influenzæ was recovered from the conjunctivæ, lungs and pleural exudate.

Rabbit 14. The animal received 2 cc. of filtrate intravenously and was placed in a cage with infected Rabbit 6. After two days he was ill; death occurring on the seventh day. At autopsy, Hemophilus influenzæ was recovered from the lungs, heart blood and pleural exudate.

The above experiments show that while normal rabbits exposed to rabbits infected with Hemophilus influenzæ, became infected and died within ten days (Experiments 9 to 12 inclusively), that rabbits which had received a sublethal dose of filtrate became infected and died in approximately seven days. Since the culture filtrates contain no Hemophilus influenzæ, the indication is that these bacteria were acquired by contact infection.

6. *Rabbits Receiving Lethal Doses of Culture Filtrate.* The lethal dose of filtrate of the Hammett strain was determined as follows. Three rabbits were injected with culture filtrate of this strain using varying doses.

Rabbit 15. Weight, 2150 gm. Injected intravenously with 2 cc. of culture filtrate (Hammett strain). The animal survived.

Rabbit 16. Weight, 2420 gm. Injected intravenously with 3 cc. of culture filtrate (Hammett strain).

Rabbit 17. Weight, 2415 gm. Injected intravenously with 5 cc. of culture filtrate (Hammett strain).

Rabbits 16 and 17. These died the day following the injections.

Necropsy. The lungs showed pin-point hemorrhages with a red serous exudate in the pleural cavity, a picture similar to the lungs of rabbits having had injections of living Hemophilus influenzæ organisms. The heart and kidneys were pale, the liver soft and friable. All cultures from organs of these rabbits were negative for Hemophilus influenzæ.

Therefore, since 2 cc. of culture filtrate did not kill and 3- and 5-cc. doses did kill when injected intravenously into rabbits, it can be concluded that 3 cc. was the M.L.D. of culture filtrate when injected intravenously.

7. *Immunization of Rabbits with Culture Filtrates of the Hammett Strain.* Three rabbits (18, 19 and 20) were immunized to the culture filtrate of the Hammett strain, giving sublethal doses at first, and increasing these doses, by giving three injections subcutaneously resting four days, and following with four courses of intravenous injections on three consecutive days and resting four days. The animals were bled and the serums of 18 and 19 gave a precipitin titer of 1 to 1280 and that of 20 of 1 to 640. Rabbit 18, immune to

filtrate when exposed to a rabbit infected with Hemophilus influenzæ, did not become infected.

An effort was made to immunize a rabbit to heated culture filtrate. Culture filtrate heated to 100° C. for one hour was used.

Rabbit 21. Received repeated injections of heated filtrate, given in the same manner as described for Rabbits 18 to 20, was exposed to an infected rabbit with open lesions. At the time of exposure, the serum of this rabbit gave a precipitin titer of 1 to 40, the precipitin titer of this same rabbit before the series of injections being negative. Rabbit 21 became ill within three days and died in eight days. At autopsy, the small bronchi were congested, the lungs were hemorrhagic and there was a serous exudate in the pleural cavity. The heart was enlarged. Hemophilus influenzæ was recovered from the heart blood, lungs and pleural exudate.

The above experiments show that rabbits can be immunized to unheated culture filtrate, and following immunization can be exposed to infected rabbits, without becoming infected. On the other hand, a rabbit receiving repeated injections of heated culture filtrate, when exposed to an infected rabbit develops the infection. It thus appears that heating a culture filtrate to 100° C. for one hour destroys its property of exciting protective antibodies in rabbits.

8. *Experiments with Culture Filtrates in Collodion Sacs.* A sealed collodion sac containing culture filtrate of the Hammett strain was placed into the peritoneal cavity of a rabbit. The experiment was performed in the following manner:

Rabbit 22. A collodion sac containing 3 cc. of brown blood-broth culture filtrate, freshly prepared, to which had been added 3 cc. of sterile brown blood broth was placed into the peritoneal cavity of the rabbit. Three months later, the serum of the rabbit showed a precipitin titer of 1 to 1280. Eight months later, the precipitin titer had fallen to 1 to 640. While the experiment was performed on only one rabbit, the opinion might be advanced that the toxic agent of the culture filtrate continued to remain active, in the collodion sac, and that the animal developed some immunity against it.

Rabbit 23. A collodion sac containing 4 cc. of brown blood broth Hemophilus influenzæ culture (Hammett strain) was placed into the peritoneal cavity of a rabbit. The next day, the animal was dead. On autopsy, it was found that the rabbit died of acute peritonitis, and that the collodion sac had broken. Pure cultures of Hemophilus influenzæ were recovered from the peritoneal pus, and the heart blood.

Conclusions on Rabbit Experiments. 1. For Hemophilus influenzæ (Hammett strain) 1 billion living organisms injected intravenously kill rabbits in eighteen hours, and produce lesions similar to those found in humans dying of influenza.

2. Living organisms of virulent strains of Hemophilus influenzæ are not as quickly fatal when injected subcutaneously as cultures injected intravenously, since chronic abscesses are formed in the former procedure from which pure cultures of Hemophilus influenzæ may be recovered several months after the initial injection.

3. Attempts at immunization of rabbits with a virulent strain of Hemophilus influenzæ failed, due to the pathogenicity of the organism. With the methods employed, the animals lost weight and local abscesses were formed when an attempt was made to administer living organisms. The agglutinin titer of the serum was only 1 to 320.

4. Normal rabbits may become infected when brought in contact with rabbits previously infected with Hemophilus influenzæ and having open lesions.

5. Rabbits previously injected with a sublethal dose of culture filtrate, will, when immediately exposed to an infected rabbit, acquire the infection and succumb sooner to this infection than would a normal rabbit if exposed to an infected rabbit. The culture filtrate appears to contain a toxic agent, which promotes infection with Hemophilus influenzæ, since infection took place sooner when a sublethal dose of filtrate was administered before exposure. The theory may be advanced that a toxic agent is present in culture filtrates of virulent strains of Hemophilus influenzæ and that it may act as an aggressin to infection with the organism.

6. Culture filtrates of a virulent strain of Hemophilus influenzæ, when injected intravenously into rabbits, can cause death. Parker[2] and Wollstein[3] also found that Hemophilus influenzæ produced a filtrable poison which was lethal for rabbits when injected intravenously.

7. Rabbits may be immunized to culture filtrates and when exposed later to infected rabbits, be immune to infection. Parker[2] also immunized rabbits to at least four or five times the M.L.D.

8. Culture filtrates heated to 100° C. for one hour previous to injection failed to immunize rabbits to the point of protection when exposed to infected rabbits. Parker[2] also found that filtrate poison when heated to 55° C. for one-half hour was partly destroyed, and when heated to 75° C. for one hour, or boiled for five minutes, over two-thirds the toxicity had been lost.

9. When a collodion sac containing culture filtrate was placed in the peritoneum of a rabbit, the serum developed a precipitin titer as high as 1 to 1280.

(*d*) **Biologic Studies on Hemophilus Influenzæ.** The biologic characteristics of all strains used in this study were determined, that is, the Hammett strain, the animal passage cultures of the Hammett strain and the strains isolated from other patients.

1. All strains plated to ascertain if the peculiar mouse-like odor was present gave positive results.

2. Hemolysis: Not any of the strains studied showed hemolysis in blood-broth cultures.

3. Litmus milk reactions: None of the strains studied gave any change in litmus milk after seven days at 37.5° C.

4. Association with Staphylococcus aureus: All strains studied

showed definite increase in the size of the colonies when grown in association with Staphylococcus aureus on red blood agar plates.

5. Indol formation: The Hammett strain and the animal passage strain originating therefrom all produced indol.

Of twenty-two other strains isolated from patients, fourteen produced indol, and eight were negative. Of the fourteen strains producing indol, only seven were virulent. The virulence of a strain and its ability to form indol are apparently not closely related.

6. Growth on plain infusion agar without blood: All strains were plated on plain beef infusion agar, pH 7.6, and none gave growth.

The above tests show that typical cultures of Hemophilus influenzæ were used for the experiment, and, as has also been shown by Rivers[4,5] that no biologic classification could be made of them. Table II shows the biologic characteristics of twenty-three strains of Hemophilus influenzæ. The fifteen animal passage generations of the original Hammett strain gave the same cultural characteristics of the parent strain.

(*e*) **Serologic Reactions.** Each of the strains of Hemophilus influenzæ included in this study was tested by agglutinative and precipitin reactions with immune serum (horse) of the Hammett strain. The technique employed in these tests was as follows:

1. *Agglutinative Reactions.* The agglutination technique followed was according to the method of Small and Evans.[6] The cultures of Hemophilus influenzæ were streaked on brown blood agar plates and these plates were incubated over night at 37.5° C. The resulting growth was washed off in infusion broth. The suspensions were first centrifugalized to throw down agar particles and then centrifugalized at high speed for forty-five minutes to sediment the bacteria. The supernatant fluid was decanted, and the sediment of microörganisms resuspended in infusion broth, and heated at 85° C. for one hour. The suspension was then standardized to 1 billion microörganisms per cubic centimeter and agglutination tests performed with immune serum diluted 1 to 200. This made the final dilution 1 to 400. The tests were incubated in a water bath at 50° C. for eighteen hours. Naked eye readings were made.

2. *Precipitin Reactions.* The precipitin technique used was according to the following method: Eighteen-hour brown blood-broth cultures were filtered through a grade N. Berkefeld filter and the filtrate was tested for sterility. Immune serum (horse) of the Hammett strain was diluted from 1 to 10 up to 1 to 2560. Equal amounts of culture filtrate was added to the diluted serum and the tests remained at room temperature for eighteen hours. Naked eye readings were made of the precipitation next morning.

Table II shows the precipitin and agglutinative reactions of the twenty-three strains of Hemophilus influenzæ studied.

The homologous strain and each of its animal passage subcultures were all agglutinated by the immune horse serum of the

Hammett strain. Of the twenty-two strains from other patients eighteen were agglutinated and four were not agglutinated. Thus, there was a high percentage of immunologic relation of these strains studied.

TABLE II.—CULTURAL CHARACTERISTICS, PRECIPITIN, AGGLUTINATIVE REACTIONS AND VIRULENCE OF TWENTY-THREE STRAINS OF HEMOPHILUS INFLUENZÆ.

Strain.	Source.	Associated with Staphylococcus aureus.	Odor.	Growth on plain infusion agar.	Indol.	Litmus milk.	Hemolysis.	Agglutination with immune serum of the Hammett strain.	Precipitin formation with immune serum of Hammett strain.	Virulence.
Hammett	Sputum	+	+	−	+	−	−	+	+	+
F 1 . .	Sputum	+	+	−	−	−	−	+	+	+
F 2 . .	Sputum	+	+	−	+	−	−	+	−	−
F 3 . .	Sputum	+	+	−	−	−	−	+	+	+
F 4 . .	Sputum	+	+	−	−	−	−	+	+	+
F 5 . .	Sputum	+	+	−	+	−	−	−	−	−
F 6 . .	Sputum	+	+	−	−	−	−	−	−	+
F 7 . .	Sputum	+	+	−	+	−	−	+	−	−
F 8 . .	Sputum	+	+	−	+	−	−	+	−	−
F 9 . .	Sputum	+	+	−	+	−	−	+	−	−
F10 . .	Sputum	+	+	−	−	−	−	+	+	+
F11 . .	Sputum	+	+	−	+	−	−	+	−	−
F12 . .	Bronchoscopic sputum	+	+	−	+	−	−	−	−	+
F13 . .	Bronchoscopic sputum	+	+	−	−	−	−	−	−	−
F14 . .	Bronchoscopic sputum	+	+	−	−	−	−	+	−	−
F15 . .	Bronchoscopic sputum	+	+	−	+	−	−	+	−	−
F16 . .	Spinal fluid	+	+	−	+	−	−	+	+	+
F17 . .	Spinal fluid	+	+	−	+	−	−	+	+	+
F18 . .	Spinal fluid	+	+	−	+	−	−	+	+	+
F19 . .	Spinal fluid	+	+	−	+	−	−	+	+	+
F20 . .	Pleural fluid	+	+	−	−	−	−	+	−	−
F21 . .	Pleural fluid	+	+	−	+	−	−	+	+	+
F22 . .	Nasal culture	+	+	−	+	−	−	+	+	+
		23+	23+	23−	15+	23−	23−	19+	11+	13+

The homologous strain and each of the cultures recovered from animal passage of this strain, gave positive precipitin reactions with its immune serum up to a dilution of 1 to 640.

Of the twenty-two other strains ten gave precipitin reactions with this serum and twelve gave negative results. In order to correlate the serologic reactions with the virulence of the several

strains, the results of the virulence test are set down in Table II for each strain.

It will be noted that eighteen of the twenty-two other strains showed agglutination. Of these eighteen strains ten gave precipitin reactions and all of these ten were virulent. Each of these ten strain gave positive precipitin reactions with the immune horse serum (Hammett strain) in dilutions of 1 to 160. None were higher.

Thus it would appear that the precipitinogens are present and are related to the toxic agent in the culture filtrate of virulent strains that agglutinate with the immune horse serum.

(*f*) **Therapeutic Application of Hemophilus Influenzæ Immune Serum.**

1. *Serum Preparation and Titration.* Because of extreme toxicity of the Hammett strain, this culture was selected to immunize a horse, in an effort to prepare an immune serum in quantities sufficient for adequate therapeutic tests in patients presenting clinical influenza.

In immunization of the horse difficulties were encountered due to the high pathogenicity of the organism. At first small amounts of killed microörganisms suspended in physiologic salt solution were injected subcutaneously. Later, living microörganisms were given subcutaneously. The injection of viable cultures subcutaneously produced abscesses and it was discontinued in favor of killed microorganisms suspended in unheated culture filtrate. Again with this combination, large abscesses were formed which when cultured were sterile for such bacteria as Staphylococci, Streptococci, or Hemophilus influenzæ. After these attempts treatment was again instituted with killed organisms suspended in physiologic salt solution. After eighteen months the serum gave an agglutinin titer of 1 to 2560 and a precipitin titer of 1 to 640.

The protective value of the immune serum has been tested on mice as follows. Killing doses, $\frac{1}{8}$ billion organisms per cc. of the Hammett strain were given intraperitoneally, followed in thirty minutes by 2 cc. of immune serum. Experiments have shown that 2 cc. of immune serum has the value of protecting mice against five killing doses of the microörganism. Controls were run on these experiments to be sure that the quantity of organisms would actually kill.

The serum has also been tested in the same way with other strains that agglutinated with the immune serum and here high protection was also manifested.

Because of the demonstrable antibodies in this serum which are capable of protecting mice against lethal doses of Hemophilus influenzæ, (that is, the Hammett strain, and other virulent strains which show positive precipitin against the immune serum) it was decided to apply it clinically in typical acute influenza to test whether it had any effect on the toxemia of this disease.

The serum has been collected from five bleedings of the horse,

approximately eight liters each and has been preserved in ampules and stored for further favorable opportunity for use. The horse is continued under treatment in an effort to maintain a high-grade of immunity.

2. *Therapeutic Use of the Serum.* Opportunity for such testing was limited to 3 cases of typical acute influenza of the respiratory type, and 4 of meningitis due to Hemophilus influenzæ.

Case Reports. *Influenza, Respiratory Type.* Only 3 cases with typical acute influenza within the first forty-eight hours of the disease were available for serum treatment.

Case I.—(Patient of Dr. Diehl.) Male, colored, aged forty-two years, complained of soreness throughout body, cough and expectoration, and extreme pain in the eyeballs. The attack occurred in the morning, accompanied by chills. In the afternoon, when brought to the hospital, he had a temperature of 102.5° F. and was acutely ill. Sputum cultures showed Hemophilus influenzæ predominating, accompanied by Streptococcus mitior. The white blood cell count was 4300. A clinical diagnosis of influenza was made. Twenty cubic centimeters of influenzæ immune serum were given intramuscularly at 5 P.M. Next morning by 11 A.M. the temperature had dropped to 98.5° F., and the white blood cell count was 9800. For twenty-four hours, the temperature continued normal and the white blood cell count was 7500. The patient felt well and continued to feel so until discharged from the hospital ten days later. The noticeable point in the convalescence of the patient was the feeling of well being in contrast to the usual symptoms of weakness and depression following an attack of influenza.

Case II.—(Patient of Dr. Lanyon.) Male, white, aged thirty years, employee in the hospital, complaining of soreness throughout the body, accompanied by chills and having a temperature of 103° F. A white blood cell count showed a leukopenia of 4000. Nasal cultures showed pure culture of Hemophilus influenzæ and throat cultures showed Hemophilus influenzæ predominating, accompanied by pneumococcus, Group 4 and Streptococcus mitior. Twenty cubic centimeters of influenza immune serum were given intramuscularly on the first day of illness. Next morning the temperature was normal, and the white blood cell count was 8500. The patient felt very well and continued to do so until his return to work. He also had none of the symptoms following influenza, such as weakness and depression.

Case III.—(Patient of Dr. Lanyon.) A young adult male with a clinical diagnosis of influenza, accompanied by high temperature and a leukopenia. Twenty cubic centimeters of influenza serum were given on the first day of illness intramuscularly followed by recovery of the patient within twenty-four hours.

Meningitis Due to Hemophilus Influenzæ. Four patients with meningitis were also treated. They were young children showing pure culture of Hemophilus influenzæ in the spinal fluid. The immune serum was given intravenously and intraspinally, and although brief, temporary improvement was reported by the clinicians, all cases proved fatal. A fourth patient, an eighteen-month-old boy, showing pure culture of Hemophilus influenzæ in the spinal fluid, was

treated with the serum. A trephine operation was performed and the spinal canal washed with serum. The serum was also administered intravenously. This patient lived for twelve days following the beginning of serum treatment. On the twelfth day the patient died.

The application of the antiserum in these few cases is presented simply as a matter of record and for the sake of completeness in reporting the work undertaken. No inference as to any possible therapeutic value is implied. Our purpose has been that of preparing a potent antiserum against a very virulent strain of Hemophilus influenzæ and storing it in sufficient quantity to test it out in clinical influenza should this disease return in epidemic prevalence. Only in this manner can it be determined whether the clinical toxemia of epidemic influenza is in any way related to the toxic agents in cultures of Hemophilus influenzæ.

Conclusions. 1. Virulence of strains of Hemophilus influenzæ may be maintained by keeping the cultures in suitable fluid media at a room temperature of 18° to 20° C. Of the twenty-three strains studied thirteen were virulent, and ten were avirulent to laboratory animals.

2. Rabbits and mice succumb to inoculation with virulent strains of Hemophilus influenzæ. They also succumb to injections of culture filtrates of virulent strains. Thus the toxic agent causing the virulence of the strain is found in the culture filtrate.

3. Normal rabbits exposed to infected rabbits become infected by contact and death ensues in ten days with characteristic lesions found at autopsy. It also appears that previous treatment of a rabbit with a sublethal dose of culture filtrate hastens the contact infection of that rabbit with Hemophilus influenzæ when it is exposed to an infected rabbit.

4. Skin reactions may be performed on human beings with culture filtrate of a virulent strain, giving positive results with a dilution of 1 to 7000. This test seems to be of the nature of the Dick or Schick reactions, and might perhaps be used to determine susceptibility to infection of humans with Hemophilus influenzæ.

5. Precipitin and agglutinin reactions have been performed on the twenty-three strains studied, using the antiserum prepared with the Hammett strain. Nineteen agglutinated, eleven of which gave a precipitin reaction with the immune serum. Only the strains that gave both positive agglutinin reactions with the immune serum and were also virulent gave precipitin reactions. Precipitin reactions apparently shows a strict parallelism to the virulence in strains of Hemophilus influenzæ.

6. The serum of a horse immunized with a virulent strain (Hammett) was found to protect mice against four to five lethal doses of Hemophilus influenzæ. A few trials of the serum in patients are recorded.

7. It would appear that infection with Hemophilus influenzæ is in direct relation with the pathogenicity of the strain, and that the pathogenicity is dependent upon the association of the organism with a toxic agent which may readily be identified in culture filtrate.

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